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Oral dependent-dose toxoplasmic infection model induced by oocysts in rats: Myenteric plexus and jejunal wall changes

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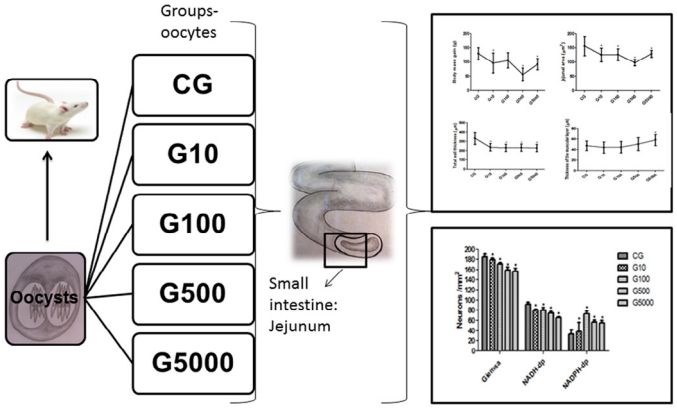
HIGHLIGHTS

- We analyzed the jejunum of rats infected with different doses of *T. gondii*.
- The tested dose was from 10 to 5000 oocysts orally.
- NADHd-p and NADPHd-p neuronal atrophy was observed.
- Morphological and morphometric alterations were found in the infected groups.
- Hypertrophy of the muscle was observed in the group infected with 5000 oocysts.

GRAPHICAL ABSTRACT



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ABSTRACT

Toxoplasmosis is a widely distributed disease caused by the protozoan *Toxoplasma gondii* that is mainly transmitted orally. Once ingested, the parasite crosses the intestinal barrier to reach the blood and lymph systems to migrate to other regions of the host. The objective of this study was to evaluate the changes in the myenteric plexus and the jejunal wall of Wistar rats caused by oral infection with *T. gondii* oocysts (ME-49 strain). Inocula of 10, 100, 500 and 5000 oocysts were used. The total population of myenteric neurons and the most metabolically active subpopulation (NADH-diaphorase positive – NADH-dp) exhibited a decrease proportional to the dose of *T. gondii*. There was also a quantitative increase in the subpopulation of NADPH-diaphorase-positive (NADPH-dp) myenteric neurons, indicating greater expression of the NOS enzyme. Neuronal atrophy was observed, and morphological and morphometric alterations such as jejunal atrophy were found in the infected groups. Hypertrophy of the external muscle with the presence of inflammatory foci was observed in the group infected with 5000 oocysts. The changes observed in the infected groups were proportional to the number of oocysts inoculated.

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1. Introduction

Toxoplasmosis is a widely distributed disease caused by *Toxoplasma gondii*, an obligate intracellular parasite in the phylum Apicomplexa, family Sarcocystidae (Grigg and Sundar, 2009; Kim and Weiss, 2004; Weiss and Kim, 2011). *T. gondii* has a heteroxenic cycle using felids as its definitive hosts and humans and other homeothermic animals as intermediate hosts (Dubey and Jones, 2008; Hill and Dubey, 2012). Intermediate hosts are mainly infected orally via ingestion of uncooked meat containing tissue cysts or oocysts present in contaminated water or food (Dubey and Beattie, 1988; Dubey and Jones, 2008; Weiss and Kim, 2011).

During chronic infection, *T. gondii* tissue cysts predominate in nervous and muscle tissues (Hill and Dubey, 2012). However, the parasite must cross the intestinal barrier to reach these tissues. During this invasion, the interaction between the host and the parasite can result in intestinal disorders that culminate in diarrhea and inflammation (Barragan and Hitziger, 2008).

Our research group was the first to show the effects of *T. gondii* infection on the enteric nervous system (ENS). Our previous studies have shown that the infection can cause changes in enteric neurons and in intestinal wall (da Silva Pde et al., 2010; Hermes-Uliana et al., 2011; Odorizzi et al., 2010; Sant'Ana et al., 2012; Shiraishi et al., 2009; Sugauara et al., 2008, 2009). These changes depend on the parasitic stage ingested, the duration of the infection, the host species and the part of the gastrointestinal tract evaluated. We hypothesize that the tissue response is dependent-dose to the parasite in chronic infections. Thus, this study is the first to show that there is a direct relationship between parasitic dose and alterations in the myenteric neurons and jejunal wall.

2. Materials and methods

2.1. Experimental design

The experimental protocol was conducted in accordance with international standards of ethical conduct in experiments and was previously approved by the Committee on Ethical Conduct in the Use of Experimental Animals (Comitê de Conduta Ética no Uso de Animais em Experimentação – CEAE) of the State University of Maringá (Universidade Estadual de Maringá – UEM) (document number 081/2012).

Male Wistar rats (*Rattus norvegicus*) (233.61 ± 5.26 g) were randomly distributed into five groups of seven animals each ($p > 0.05$). The work follows a fully randomized experiment design.

Blood samples were collected by puncture of the retro-orbital plexus before and after inoculation of *T. gondii* oocysts to check for the presence of serum anti-*T. gondii* IgG antibodies using the direct agglutination test, with samples with titers above 25 considered positive (Desmonts and Remington, 1980).

2.2. Experimental infection

The control group (CG) received sterile 0.9% NaCl aqueous solution, and the infected groups received a solution containing 10 (G10), 100 (G100), 500 (G500) or 5000 (G5000) sporulated oocysts via gavage. The animals were maintained in individual cages in a maintenance room with controlled temperature and humidity, a light–dark cycle of 12 h and food and water *ad libitum*. The ME-49 strain oocysts (genotype II) used in this study was obtained from the Laboratory of Veterinary Parasitology of the Londrina State University (Universidade Estadual de Londrina – UEL). These oocysts were originally isolated from sheep muscle in the USA (Lunde and Jacobs, 1983).

2.3. Euthanasia and collection of biological material

After 30 days of infection, the animals were euthanized under deep anesthesia with halothane vapor (Tanohalo®, Cristália, Itapira, São Paulo, Brazil) (Vivas et al., 2007). Vertical laparotomy was performed, and the jejunum was removed and measured. The duodenojejunal flexure (proximal) and the ileocecal fold (distal) were used as anatomical references for measurement of the jejunum. The proximal jejunum was used for the analysis.

2.4. Analysis of the intestinal wall

A 2-cm ring of the jejunum was collected and subjected to routine histological examination. Semi-serial 4 μm transverse sections were cut and stained with hematoxylin and eosin (HE).

Morphometric analysis of the intestinal wall was performed from images captured by a digital camera (Pro series 3CCD camera) coupled to an optical microscope (Olympus BX50). Images captured with the 4× objective lens were used to measure the total thickness of the intestinal wall, and images captured with the 20× objective lens were used to measure the muscular tunic. Each histological section was divided into four quadrants, and each quadrant was measured along the circumference. Four sections from each animal in each group were analyzed, totaling 16 measurements per animal. The analyses were performed with the aid of the Image Pro Plus software (Media Cybernetics).

2.5. Analysis of the myenteric plexus

With the aid of a stereomicroscope with transillumination (Olympus Micronal SZ40) the jejunum was microdissected with the removal of the tunica mucosa and the tela submucosa. The whole mounts formed by the muscular and serous tunics were subjected to the following techniques: Giemsa stain (Barbosa, 1978), to show the total neuronal population; NADH-diaphorase (NADH-d) (Gabella, 1969), to mark the metabolically more active subpopulation; and NAPDH-diaphorase (NADPH-d) (Scherer-Singler et al., 1983) to show the subpopulation that produces nitric oxide.

2.5.1. Giemsa staining

A 5-cm jejunum segment was washed in NaCl solution (0.9%), filled and immersed in acetic formalin fixative solution for 48 h. The jejunum was cut into samples of roughly 1 cm length to prepare whole mounts of the myenteric plexus. The whole mounts were Giemsa stained using a protocol that is based on the visualization of neurons by methylene blue (Barbosa, 1978).

2.5.2. NADH-d histochemistry

Jejunum segments (5 cm) was washed with Krebs buffer (pH 7.3) and immersed for 5 minutes in 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in Krebs buffer. Afterwards, the jejunum segments were washed twice (10 minutes each wash) with Krebs solution and immersed for 45 min in a solution containing the following per 100 mL: 0.05 g of β-NADH (Sigma, St. Louis, MO, USA), 25 mL of nitroblue tetrazolium (NBT) stock solution (Sigma, St. Louis, MO, USA), 25 mL of 0.1 M phosphate buffer (pH 7.3) and 50 mL of distilled water (Gabella, 1969). The reaction was stopped by the addition of 10% buffered formalin.

2.5.3. NADPH-d histochemistry

Approximately 5 cm of the jejunum was washed with 0.1 M PBS (pH 7.3), immersed for 30 min in 4% buffered paraformaldehyde (Sigma, St. Louis, MO, USA), immersed for 10 min in 0.3% Triton X-100 (Sigma, St. Louis, MO, USA) in 0.1 M PBS and washed 10 times (10 min each wash) with 0.01 M PBS solution. Subsequently, the sections were immersed for 2 h in a solution containing the following

per 100 mL: 0.05 g of β-NADPH (Sigma, St. Louis, MO, USA), 0.6 mL of Triton X-100 and 0.05 g of NBT (Sigma, St. Louis, MO, USA) in a total volume of 200 mL of Tris–HCl solution (Life Technologies, Grand Island, NY, USA) (Scherer-Singler et al., 1983). The reaction was stopped by the addition of 4% paraformaldehyde.

2.5.4. Quantitative analysis of the myenteric plexus

Each whole mounts was divided into three regions: mesenteric, intermediate and antimesenteric. Neurons present in 120 microscope fields were counted (40 fields per region), totaling an area of 26.4 mm^2 per animal. For the analysis, an optical microscope (Olympus BX50) with a 40× objective lens was used. Neurons located within the limits of each field were counted in alternate fields.

2.5.5. Morphometric analysis of the myenteric plexus

The areas of the cell body, cytoplasm and nucleus of 300 myenteric neurons (100 neurons from each of the three regions of the jejunum) of each animal were measured with the Image Pro Plus software (Media Cybernetics). The measurements were performed with the aid of a microscope (Olympus BX50) equipped with a digital camera (Pro series 3CCD camera) connected to a computer.

2.6. Statistical analysis

The data had a normal distribution by the D'Agostinho–Pearson test and are presented as the mean ± standard deviation. Comparisons between groups were performed using test T, and the results were considered significantly different when $p < 0.05$. The statistical analyses were performed using the BioEstat 5.3 software (Ayres et al., 2007).

3. Results

The animals of the infected groups were positive for anti-*T. gondii* IgG, whereas the animals of the control group remained negative. The infected animals, with exception of the G100, had a lower amount of body weight gain and a smaller jejunum area than the CG ($p < 0.05$) (Fig. 1).

Morphometric analysis of the intestinal wall showed that the infection caused atrophy of the jejunal wall in the infected groups ($p < 0.05$) (Fig. 1). The animals infected with 5000 oocysts showed a 23% increase in hypertrophy of the muscular tunic ($p < 0.05$) (Figs. 1 and 2). Leukocyte infiltration and *T. gondii* tissue cysts were also observed in the animals infected with 5000 oocysts (Fig. 2).

Losses of 3.32%, 7.42%, 13.89% and 15% of the total population of myenteric neurons were observed after inoculation of 10, 100, 500 and 5000 oocysts, respectively. There was also a progressive reduction in the NADH-d-positive (NADH-dp) subpopulation; the inoculation of 10, 100, 500 and 5000 oocysts resulted in losses of 12%, 12.13%, 19.75% and 27.93%, respectively (Fig. 3). Cellular atrophy was observed using both techniques ($p < 0.05$). However, there was an increase in the number of NADPH-d-positive (NADPH-dp) neurons in the infected groups compared with the control; the increase ranged from 14% (G10) to 67% (G5000). In addition, hypertrophy

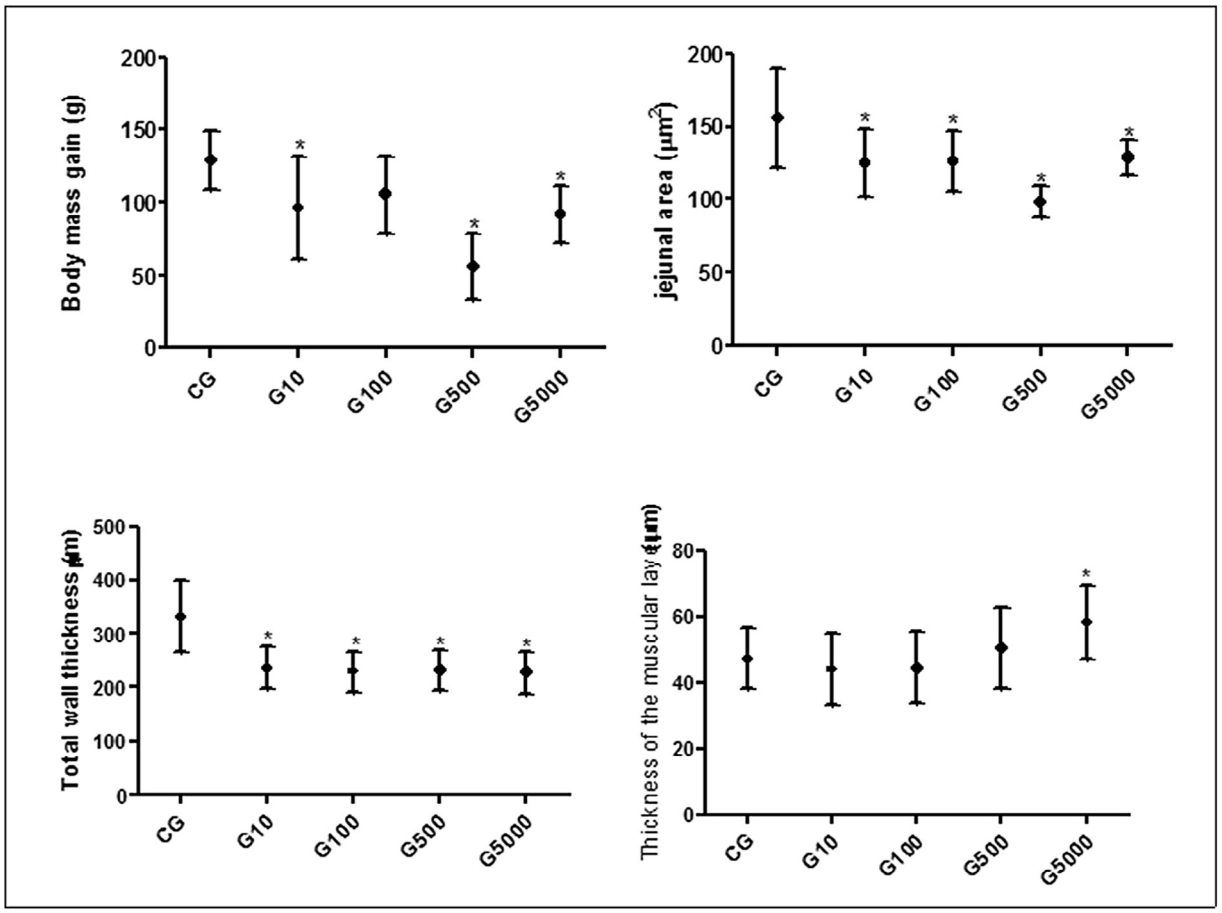

Fig. 1. Body weight gain, total jejunal area, total wall thickness and muscular tunic thickness of rats infected with different inocula of *T. gondii* for 30 days. Means ± standard deviations marked with * indicate significant differences ($p < 0.05$) compared with the CG by test T.

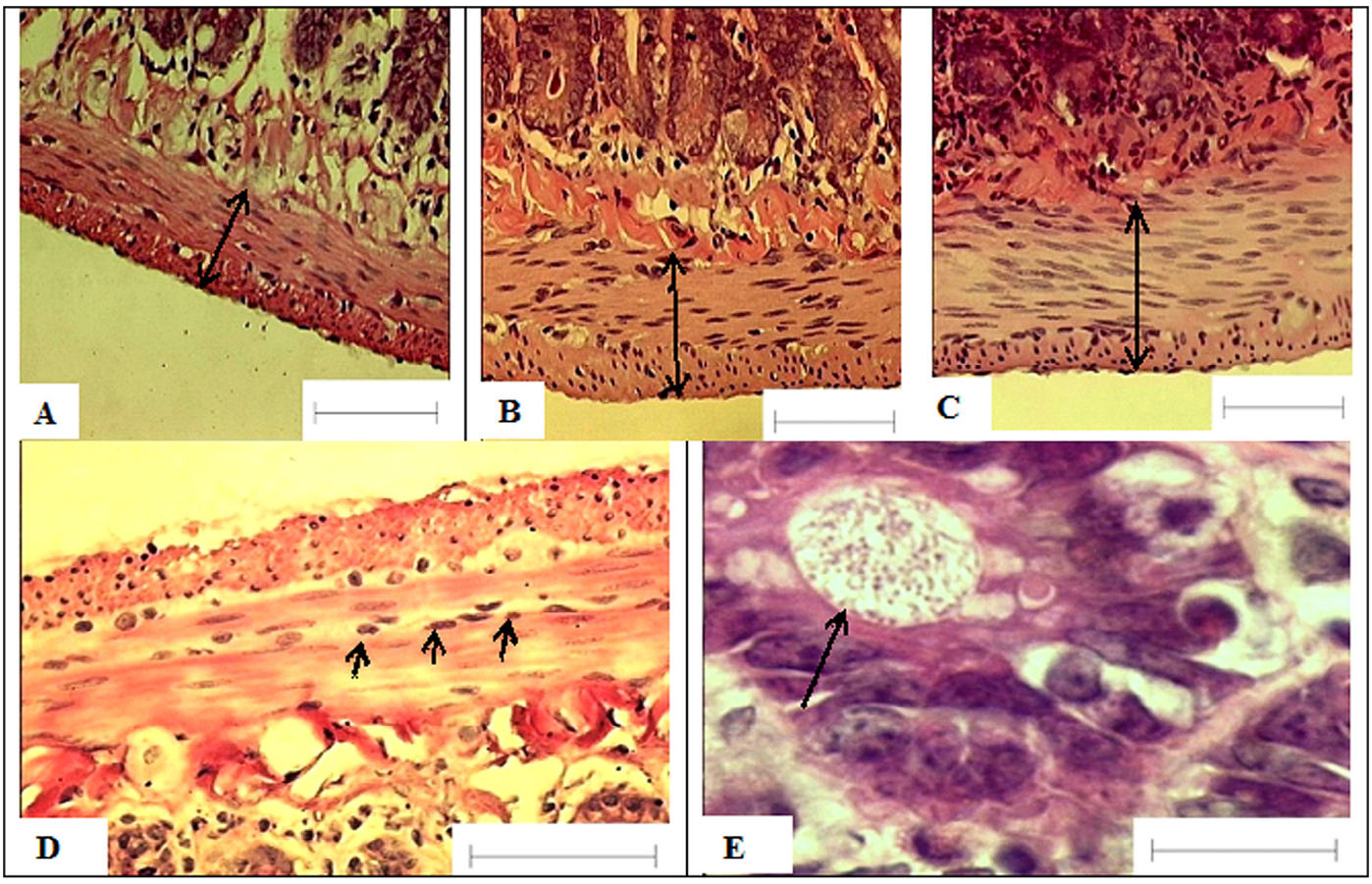

Fig. 2. Micrograph of transverse cuts of the jejuna of rats in the CG (A) and in the groups infected with 500 (B) and 5000 (C, D, E) oocysts of *T. gondii* for 30 days showing the muscular tunic (line) and hypertrophy in the infected groups ($p < 0.05$). 200× magnification. (D) Presence of inflammatory cells in the circular muscular tunic (arrows). (E) Presence of *T. gondii* cysts in the lamina propria (arrow). 1000× magnification. Bar: 50 μm. HE.

of the soma and cytoplasm in the G10 and cellular atrophy in the other infected groups was observed (Table 1, Fig. 4).

4. Discussion

In the present study, *T. gondii* led to subclinical infection without the development of symptoms such as fever, diarrhea and death, as occurs in the majority of infections of immunocompetent humans. Rats are immunocompetent and are usually resistant to infection by *T. gondii* (Dubey, 1998; Dubey and Frenkel, 1998), but the disease may become chronic, followed by formation of tissue cysts. The ME-49 strain has intermediate virulence and cystogenic potential (Maubon et al., 2008). Therefore, the interaction between a resistant host and a cystogenic strain led to the observation of *T. gondii* cysts in the intestinal wall, although this condition is rare (Dubey and Beattie, 1988). We emphasize that although 30 days of infection elapsed, the presence of tissue cysts in the intestine may still have produced an active immune response, thus contributing to the onset of intestinal inflammation (Kumar, 2011).

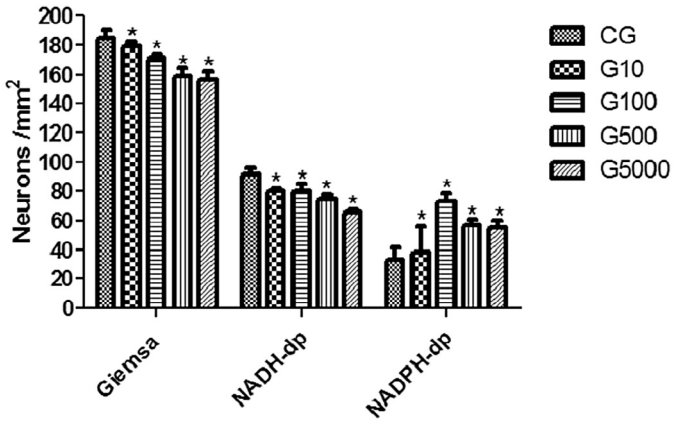

Fig. 3. Number of neurons/mm^2 in the jejuna of rats infected with *T. gondii* oocysts (ME-49 strain) for 30 days and visualized by Giemsa staining and NADH-d and NADPH-d histochemistry. (*$p < 0.05$ for each technique compared with the CG by test T.)

Oral infection by different inocula of *T. gondii* oocysts (ME-49 strain) induced atrophy of the jejunal wall and possibly caused the smaller total jejunal area in the infected animals. Our research group has demonstrated similar results in previous studies of *T. gondii* infection in the duodenum of cats (ME-49 strain (da Silva Pde et al., 2010)) and in the ileum of chickens (M7741 strain (Shiraishi et al., 2009)).

However, there was hypertrophy of the muscular tunic in the G5000 that led us to question the mechanism of this change. One hypothesis is that this may have occurred because the muscular tunic has an essential role in the intestinal immune response (Bauer, 2008; Weiss and Kim, 2011); between the muscle fibers, there is a network of macrophages, mast cells and lymphocytes that respond to the stimuli caused by the endotoxins of microorganisms (Bauer, 2008). When activated, these immune cells induce hyper-contractility of smooth muscles to expel the parasite (Bauer, 2008). Based on this,

Table 1
Area of the soma, nucleus and cytoplasm and nucleus/soma ratio of myenteric neurons of the jejuna of rats infected with different inocula of *T. gondii* (ME-49 strain) for 30 days as visualized by Giemsa staining and NADH-d and NADPH-d histochemistry.

	Cell body area (μm²)	Nucleus area (μm²)	Cytoplasm area (μm²)	Nucleus/cell body ratio
Giemsa				
CG	173.73 ± 63.90[a]	77.01 ± 2.74[a]	96.71 ± 43.82[a]	0.45 ± 0.08[a]
G10	150.16 ± 64.31[b]	69.18 ± 24.71[b]	80.97 ± 44.65[b]	0.48 ± 0.09[b]
G100	147.04 ± 60.76[c]	62.19 ± 22.72[c]	84.84 ± 42.87[c]	0.43 ± 0.08[c]
G500	149.29 ± 40.78[d]	66.29 ± 18.03[d]	82.99 ± 29.30[d]	0.45 ± 0.08[a]
G5000	147.33 ± 55.44[e]	57.54 ± 18.85[e]	89.79 ± 42.52[e]	0.41 ± 0.09[d]
NADH-dp				
CG	193.00 ± 67.68[a]	86.02 ± 27.48[a]	107.86 ± 49.15[a]	0.46 ± 0.01[a]
G10	149.56 ± 68.16[b]	63.82 ± 27.94[b]	85.3 ± 47.71[b]	0.44 ± 0.01[b]
G100	156.83 ± 70.06[c]	57.65 ± 26.97[b]	99.17 ± 53.40[c]	0.38 ± 0.10[c]
G500	158.72 ± 68.60[d]	71.16 ± 28.01[c]	87.56 ± 48.77[bd]	0.47 ± 0.12[d]
G5000	154.40 ± 73.72[e]	62.25 ± 26.66[b]	92.14 ± 55.18[d]	0.43 ± 0.11[e]
NADPH-dp				
CG	193.47 ± 62.66[a]	81.19 ± 25.43[a]	112.27 ± 50.28[a]	0.43 ± 0.10[a]
G10	201.83 ± 65.43[b]	90.66 ± 29.08[b]	111.16 ± 50.48[a]	0.46 ± 0.11[b]
G100	183.63 ± 53.21[c]	77.91 ± 19.86[c]	105.72 ± 42.47[b]	0.43 ± 0.09[a]
G500	172.63 ± 49.48[d]	77.01 ± 19.73[c]	95.61 ± 37.80[c]	0.45 ± 0.09[b]
G5000	175.18 ± 51.60[d]	85.63 ± 20.76[d]	89.55 ± 38.76[d]	0.50 ± 0.09[c]

Means ± standard deviations followed by different letters in the same column for the same technique indicate significant differences ($p < 0.05$) by test T.

the findings of the present study may be a reflection of hypertrophic and/or hyperplastic changes.

In previous studies, inflammatory infiltrates were observed in the intestinal wall of birds (Bonapaz et al., 2010), domestic cats (da Silva et al., 2010) and mice (Rachinel et al., 2004) infected with *T. gondii*, along with the predominant presence of eosinophils (Bonapaz et al., 2010), demonstrating the ability of this parasite to induce inflammation in the host intestine (Hermes-Uliana et al., 2011). Therefore, as reported by Liesenfeld (2002), infection by *T. gondii* in immunocompetent animals is also an important experimental model of intestinal inflammation. We believe that understanding such tissue and neuronal changes and the response of the lymphoid tissue associated with the intestine may contribute to the understanding of human inflammatory intestinal diseases that are common in immunocompetent individuals. The question remains whether there is a correlation between patients with inflammatory intestinal diseases and seropositivity to *T. gondii*.

The present study is the first to demonstrate that infection by *T. gondii* oocysts (ME-49 strain) leads to a reduction in the myenteric neuronal population that is proportional to the inoculum. Reduction in the total population of myenteric neurons was observed in previous studies from our group in the duodenum of birds (1000 oocysts – M7741 (Bonapaz et al., 2010)) and in the stomach of rats (500 oocysts – M7741 (Alves et al., 2011)). However, in other studies that used different inocula, strains and organs, no quantitative changes in the enteric nervous system were observed (Hermes-Uliana et al., 2011; Pereira et al., 2010).

There was also a reduction in neurons in the NADH-dp subpopulation in all infected groups, with a response proportional to the inocula except in G10 and G100. However, the neuronal reduction was much more dramatic in the NADH-dp subpopulation than in the total population. These results differ from Pereira et al. (2010) and Odorizzi et al. (2010), who found no neuronal reduction in this subpopulation when studying the jejunum of rats (ME-49 strain) infected for 24 hours and the jejunum of pigs (M7741 strain), respectively. Neuronal visualization using NADH-d histochemistry is based on the formation of a formazan precipitate that occurs as NBT is reduced at the expense of NADH oxidation in a reaction catalyzed by complex I of the mitochondrial electron transport chain (Berridge et al., 2005). When the NADH-d technique is used while maintaining the same incubation times for all whole mounts, as in the present study, the degree of metabolic activity of myenteric neurons from different experimental treatments can be compared (de Sousa and de Miranda Neto, 2009). Thus, the reduction in the density of NADH-dp neurons indicates that the parasitic infection negatively affected the neurons, reducing their metabolic activity and possibly compromising mitochondrial function via indirect mechanisms, such as inflammation, because *T. gondii* induces Bcl-2 overexpression in the parasitized cells. The overproduction of Bcl-2 prevents mitochondrial alterations related to cellular apoptosis (Hwang et al., 2010) and death of the parasitized cells.

For the neurons visualized by both techniques, atrophy of the soma, cytoplasm and nucleus and an increase in the proportional area occupied by the nucleus in the soma were observed. These results are similar to those found by Hermes-Uliana et al. (2011) when studying the jejunum of rats infected for 30 days with the M7741 strain. The reduction in cell area may be related to a lower metabolic activity of the cell with a lower protein synthesis supported by a smaller nuclear area. However, the reduction of the cell area may also indicate the beginning of apoptosis by these neurons, which would result in the death of a large number of cells as the infection progresses.

The quantitative increase observed in the NADPH-dp neurons differs from the results of Odorizzi et al. (2010), who did not find quantitative changes but did observe cellular hypertrophy in this subpopulation of myenteric neurons in pigs infected by *T. gondii* (M7741 strain). Hermes-Uliana et al. (2011) did not find a significant increase in the number of NADPH-dp neurons but did observe significant cellular atrophy after 30 days of infection by *T. gondii* M7741 strain. The NADPH-dp neurons, which are considered nitrergic neurons, express the enzyme nitric oxide synthase type 1 (NOS-1), and their increase may be related to increased production and release of nitric oxide (NO). This compound helps fight the parasite by preventing *T. gondii* replication and is also an inhibitory neurotransmitter in the gastrointestinal tract (Belai et al., 1995; Furness, 2008; Toole et al., 1998). The increase in NO could trigger the relaxation of smooth muscle and consequent constipation, a symptom that was not observed in the animals of the present study. It is important to emphasize that nitrergic neurons are normally protected from death (Cowen et al., 2000; Hermes-Uliana et al., 2011; Phillips et al., 2003), possibly because they have defenses against mechanisms of oxidative stress. However, the increase in the number of neurons that express NOS may result from a phenotypic shift in which the

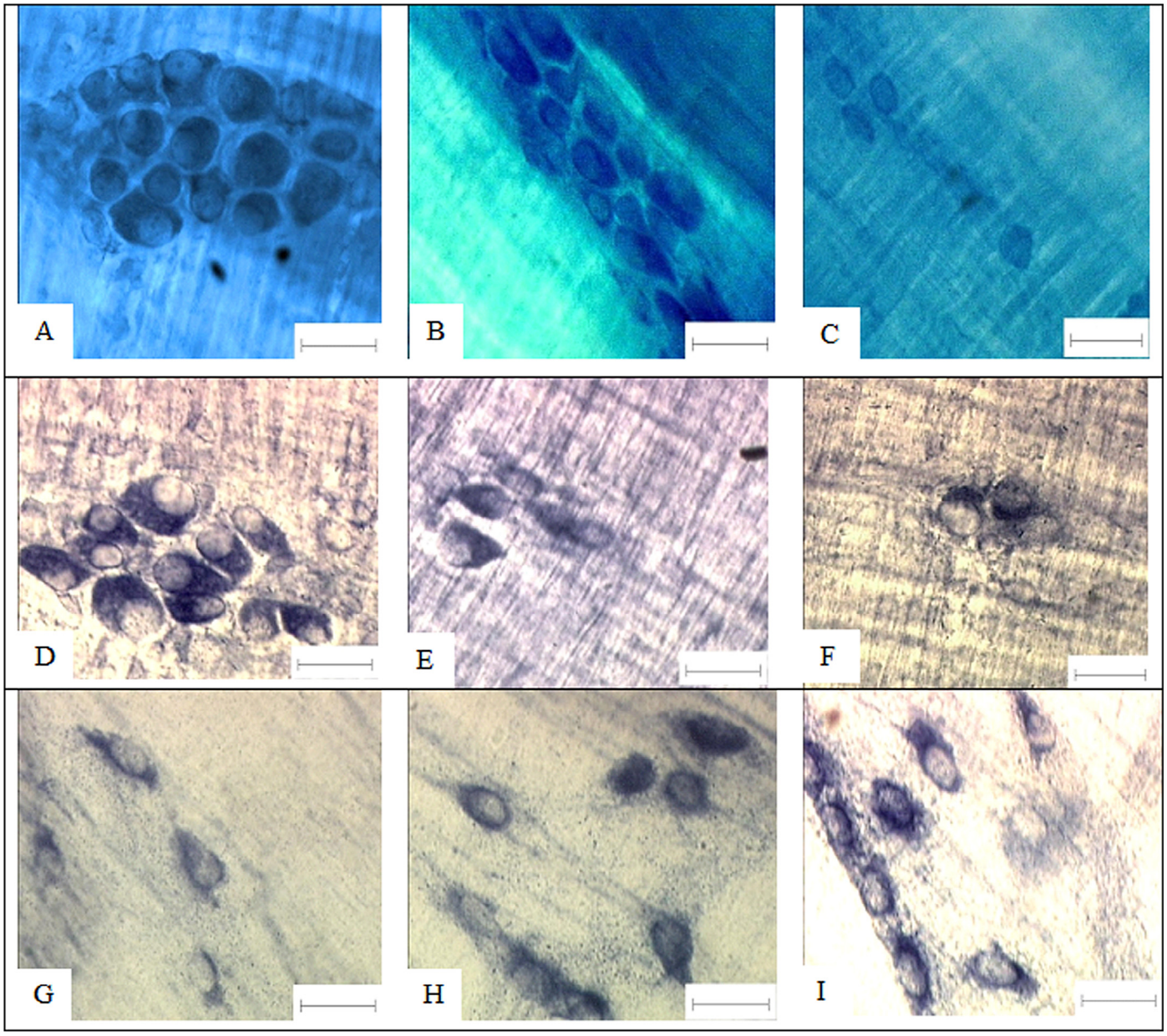

Fig. 4. Whole mounts showing ganglia of the myenteric plexus of rats of the control group (A, D, G) and rats infected with 500 (B, E, H) and 5000 (C, F, I) *T. gondii* oocysts for 30 days. Whole mounts were stained with Giemsa (A, B, C) and marked by NADH-d (D, E, F) and NADPH-d (G, H, I) histochemistry. 400× magnification. Bar: 25 μm.

neurons began to express the enzyme NADPH-d (Hermes-Uliana et al., 2011; Odorizzi et al., 2010) to help fight the parasite. Nitric oxide is cytotoxic and inhibits mitochondrial enzymes. The increase of this compound in an attempt to combat the parasite may also have affected the neurons, especially the NADH-dp subpopulation, because recent studies have demonstrated the mechanisms by which NO affects mitochondrial functioning (Park et al., 2014).

The transposition of the intestinal barrier and the chronicity of the infection with formation of local tissue cysts cause damage to the structure and proper functioning of the digestive system and enteric nervous system even with low-virulence strains and immunocompetent and resistant models. Further studies are needed to clarify the mechanisms involved in the relationship between the local immune system of the host and the parasite and the subsequent histopathological changes.

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